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Skeletogenesis

In Vitro Analysis of Bone Cell Differentiation

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1. Introduction

A common goal of cell biologists is the establishment of in vitro model systems that faithfully recapitulate a particular biological process that occurs in vivo. Currently, numerous methodologies exist for in vitro analysis of osteoblastic cells, however, many of these methods depend on the use of fetal tissue, osteosarcoma, or immortalized cell lines. Although all of these model systems have generated a great deal of knowledge on the phenotypic character of osteoblastic cells as they undergo the maturational process that ultimately leads to the formation of a mineralized matrix, a great deal of variability has been noted in the literature from one culture method to another. This variability can arise from differences in the animal species and the developmental age of the starting material used for the establishment of such cultures, the amount of soft tissue associated with the starting material, and alterations in patterns of phenotypic expression owing to tumorogenic or immortalization processes *(1)*.

The method that is described in this chapter was developed in order to minimize some of the potential differences in the starting material used to generate osteoblastic cultures *(2)*. The method employs the use of bacterial collagenase to pretreat fragments of bone to remove all soft tissue components. Whereas it is recognized that the soft tissue associated with bone surfaces such as periosteum and marrow contain osteogenic progenitor cells, they are present in variable amounts depending on the developmental age and site from which the bone is harvested. These progenitors can contribute significantly to maturational heterogeneity or expression of other phenotypes. Specific methods for establishment of such osteogenic progenitors free of other cell types (hematopoietic, endothelial) have been reported, and are described elsewhere in this volume (mesenchymal stem cells). Rather, this method focuses on cultures established from cells that are protected from the collagenase pretreatment because they are surrounded by mineralized matrix (osteocytic cells), that are subsequently able to migrate from bone fragments and begin to proliferate. The resulting cells display characteristics of preosteoblasts by virtue of alkaline phosphatase activity, formation of cAMP after treatment with parathyroid hormone (PTH), and with time in culture, they become

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mature osteoblasts that form mineralized nodules in vitro. This method can be applied to fetal and postnatal material from all animal species, including human, and has been used extensively to characterize the biosynthesis of bone matrix proteins, expression of these proteins as a function of differentiation, and other parameters of the osteoblastic lineage *(3–6)*.

2. Materials

1. Storage medium: Any nutrient medium that contains 10% fetal bovine serum (FBS).
2. Enzyme medium:
   225 mL of DMEM with 4.0 g glucose/L
   (Biofluids, Inc., Rockville, MD, list no. 104).
   225 mL Ham's F-12K without calcium chloride
   (Biofluids, Inc., list no. 161)
   5 mL glutamine (200 m*M*)
   (Biofluids, Inc., list no. 300)
   5 mL pen-strep (10,000 U/mL)
   (Biofluids, Inc., list no. 303)
   1.25 mL of L-ascorbic acid sodium salt (10 mg/mL)
   (Sigma Chemical Co., St. Louis, MO, catalog no. A-7631)
   3.9 mL of calcium stock (116 m*M*)
   (Biofluids, Inc., list no. 342)
   Filter through a 0.22-µm 500-mL filter unit.
3. Collagenase P: From *Clostridium histolyticum* (Boehringer Mannheim, Mannheim, Germany (cat no. 1213873)
   250 U of collagenase/mL
   Filter through a 0.22-µm 150-mL filter unit.
4. Growth medium: 225 mL of DMEM without calcium chloride and with 4.0 g glucose/L
   (Biofluids, Inc., list no. 160)
   225 mL of Ham's F-12K without calcium chloride
   (Biofluids, Inc., list no. 161)
   5 mL glutamine 200 m*M*
   (Biofluids, Inc., list no. 300)
   5 mL pen-strep (10,000 U/mL)
   (Biofluids, Inc., list no. 303)
   1.25 mL of L-ascorbic acid sodium salt (10 mg/mL)
   (Sigma, cat. no. A-7631).
   50 mL FBS
   Filter through a 0.22-µm 500-mL filter unit.
5. Complete medium: 225 mL of DMEM with 4.0 g glucose/L
   (Biofluids, Inc., list no. 104)
   225 mL of Ham's F-12K without calcium chloride
   (Biofluids, Inc., list no. 161)
   5 mL glutamine (200 m*M*)
   (Biofluids, Inc., list no. 300)
   5 mL pen-strep (10,000 U/mL)
   (Biofluids, Inc., list no. 303)
   1.25 mL of L-ascorbic acid sodium salt (10 mg/mL)
   (Sigma, cat. no. A-7631)
   3.9 mL of calcium stock (116 m*M*)
   (Biofluids, Inc., list no. 342)

50 mL FBS
Filter through a 0.22-μm 500-mL filter unit

6. Serum free medium:
   240 mL of DMEM with 4.0 g glucose/L
   (Biofluids, Inc., list no. 104)
   240 mL of Ham's F-12K without calcium chloride
   (Biofluids, Inc., list no. 161)
   5 mL glutamine (200 m*M*)
   (Biofluids, Inc., list no. 300)
   5 mL pen-strep (10,000 U/mL)
   (Biofluids, Inc., list no. 303)
   1.25 mL of L-ascorbic acid sodium salt (10 mg/mL)
   (Sigma, cat. no. A-7631)
   3.9 mL of calcium stock (116 m*M*)
   (Biofluids, Inc., list no. 342)
   Filter through a 0.22-μm filter unit
   then add 2.5 mL of ITS$^+$ (N.B. ITS PLUS!)
   (Collaborative Research, New Bedford, MA, cat. no. 40352)
7. Mineralization medium:
   225 mL of DMEM with 4.0 g glucose/L
   (Biofluids, Inc., list no. 104)
   225 mL of Ham's F-12K without calcium chloride
   (Biofluids, Inc., list no. 161)
   5 mL glutamine (200 m*M*)
   (Biofluids, Inc., list no. 300)
   5 mL pen-strep (10,000 U/mL)
   (Biofluids, Inc., list no. 303)
   1.25 mL of L-ascorbic acid sodium salt (10 mg/mL)
   (Sigma, cat. no. A-7631)
   3.9 mL of calcium stock (116 m*M*)
   (Biofluids, Inc., list no. 342)
   50 mL FBS
   5 mL of β-glycerol phosphate (500 m*M*)
   (Sigma, cat. no. G-6251)
   Filter through a 0.22-μm 500-mL filter unit
   then add 2.5 mL of ITS (N.B! not ITS PLUS)
   (Collaborative Research, cat. no. 40350)
8. Freezing medium:
   44 mL αMEM
   50 mL FBS
   5 mL dimethylsulfoxide
   (Sigma, cat. no. D-2650)
   1 mL pen-strep (10,000 U/mL)
   (Biofluids, Inc., list no. 303)

3. Methods

1. *Normal human trabecular bone harvest*: under sterile conditions, remove trabecular bone from the bone specimen (patella, tibia, distal femoral epiphysis, or femoral head) using a sterile No. 2 bone curet. Place scooped bone fragments into sterile Pearce Reacti-vials (Pearce Butler Co., Rockford, IL) (3-mL size) filled with 1.5 mL of enzyme media. The scooped bone chips must be keep moist throughout the procedure (*see* **Note 1**).

2. *Mincing/washing*: mince the bone chips in each vial using small sharp surgical scissors. After allowing the fragments to settle briefly, aspirate the supernatant fluid. Wash the bone fragments with enzyme medium to remove fat and bone marrow several times by adding fresh enzyme medium, allowing the fragments to settle before aspiration. Continue mincing until the fragments have a fine, sandy texture. Transfer the fragments into a sterile 50-mL conical tube containing enzyme medium (about 30 mL), and further wash the fragments by vortexing (10 times, 2 min each) to remove tissue debris. This action will further release soft tissue from the bone fragments. This is to be repeated until the enzyme medium becomes clear when looking through the tube (*see* **Note 2**).
3. *Pretreatment of bone fragments with collagenase* (*see* **Note 3**): estimate the volume of bone fragments (not more than 5 mL settled bed volume per tube), then add 50 mL of collagenase P enzyme media (1.2 mg collagenase P in 50-mL conical tube). The bone fragments are digested for 2 h on a rotator in an incubator at 37°C, or until the cellular material on the bone surface disappears (**Fig. 1A**).
4. *Plating of collagenase pretreated bone fragments*: after digestion, the fragments are washed to stop the collagenase activity by allowing to fragments to settle to the bottom of the tube and drawing off the supernatant fraction (four times, 2 min each with 30 mL of enzyme medium without the enzyme). Bone fragments are then placed into a sterile Petri dish treated for tissue culture growth (Falcon, Los Angeles, CA). One No. 5 curet filled with collagenase pretreated fragments is used to seed a 150-mm plate or one No. 2 curet for a 100-mm dish containing the growth medium. Swirl the plates around for few seconds to spread the bone chips evenly onto the Petri dish. The growth medium is replaced three times a week. The bone fragments attach to the dishes and cells should emerge after two weeks of bone preparation (**Fig. 1B**). Four to six weeks are required for the cells to reach confluence depending on the age of the donor (*see* **Notes 4–6**).
5. *Passaging*: when primary cultures become approx 70–80% confluent (**Fig. 1C**), they should be washed twice with Hank's balanced salt solution (2 min each) before adding trypsin. Five mL of freshly thawed trypsin-EDTA at 40°C should be added per 150-mm dish and the dish incubated at 37°C for approx 10 min when the majority of the cells have detached from the fragments and the dish. The cells are broken up into a single-cell suspension by drawing the trypsin up and down in a pipet several times. The cell suspension is then removed, leaving the fragments behind (or for further treatment with trypsin to remove more cells) and placed in a sterile 50-mL tube. Trypsin is inactivated by adding FBS to a final concentration of 10%. The suspension is allowed to sit for 5 min to allow any fragments that are transferred inadvertently to settle to the bottom. The supernatant fraction is carefully removed to avoid drawing up the settled fragments and the number of the cells in the suspension are counted. For proliferation studies, cells are plated at a density of 10,000 cells/cm^2, in either 24- or 48-well plates (Costar, Cambridge, MA) in serum-free or serum reduced (1–2% FBS) medium.
6. *Storage of first passage cells*: If cells are not to be used immediately, the suspension is centrifuged for 5–10 min at 100*g* to pellet the cells. The cells are resuspended in freezing medium by pipeting up and down approximately eight times. The cells are then counted by either a hemocytometer or automated methods (Coulter counter) and the volume adjusted with freezing medium to between $1–5 \times 10^6$ cells/mL. One mL of cell suspension is placed in a cryotube, which is placed into a specially designed freezing container (Nalge Nunc, Rochester, NY, cat. no. 5100-0001) with 2-propanol at room temperature. The freezing container is then transferred to a –80 freezer for 24 h, at which time the cryotubes are transferred to a liquid nitrogen container for storage. Viability of cells is maintained for at least 1 yr.

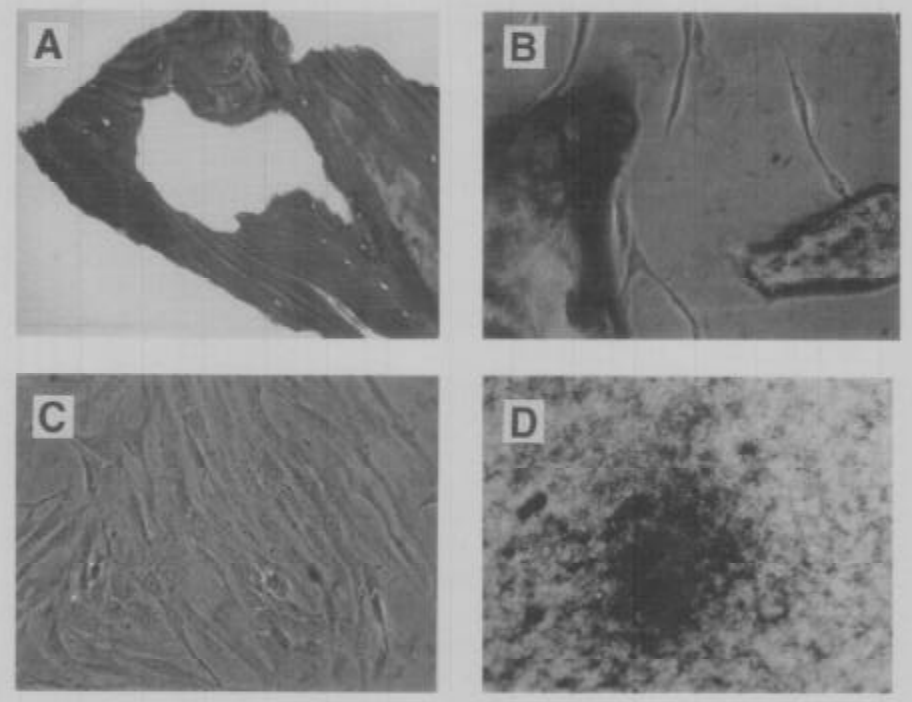

Fig. 1. Preparation of normal human trabecular bone cells for studies of osteoblastic maturation in vitro. **(A)** Small fragments of trabecular bone are minced until they reach the consistency of sand and treated with bacterial collagenase to remove all of the soft tissues associated with the mineralized matrix. **(B)** When the pretreated fragments are placed in low-calcium growth medium, cells begin to emerge from the fragments after approx 2 wk, depending on the age of the donor. **(C)** With continued incubation in low calcium growth medium, the cells form a monolayer of ellipsoid cells. **(D)** When switched to mineralization medium, the cells begin to multilayer and form bone nodules as indicated by von Kossa staining.

Table 1
Expression of Bone Matrix Proteins as a Function of Maturational Stage

Osteoprogenitor	Preosteoblast	Osteoblast	Mature osteoblast	Osteocyte
± Alkaline Phosphatase	Alkaline Phosphatase	Decorin	Osteopontin	Fibronectin
Versican	Collagens I and III	Thrombospondin	Bone Sialoprotein	Biglycan
Heparan Sulfate PGs	Decorin	Fibronectin	Collagen type I	Osteocalcin
Collagen I and III	Thrombospondin	Osteonectin		
		Biglycan		

7. *Osteoblastic differentiation*: For in vitro analysis, cells are plated at 40,000 cells per cm^2 in mineralization medium and fed three times a week. Cells proliferate, multilayer, and form bone-like nodules as has been described previously in this and other systems (**Fig. 1D**). The pattern of bone matrix protein expression by these cells in vitro ***(7–9)*** mimics what has been described by *in situ* analysis of bone formation ***(10)*** (**Table 1**). The osteoblastic

nature of the cells has also been confirmed by demonstration of bone formation using an in vivo assay (Majolagbe and Gehron Robey, unpublished data).

4. Notes

1. The bone specimen should be placed in a sterile container with nutrient medium containing 10% FBS and stored at 4°C until processed. Furthermore, the bone sample should be used within 48 h of surgery to ensure viability.
2. Proper mincing and washing is critical for the removal of soft tissue and cellular debris from the bone fragments. This step is also necessary to optimize cell growth. Removal of soft tissue is more efficient and better cellular outgrowth is obtained from small, sand-like fragments than from larger fragments.
3. Many commercial preparations of collagenase have high levels of clostripain activity, which is toxic to many cell types. Preparations with a high collagenase:clostripain activity should be selected if collagenase P is not used.
4. Using the medium formulations described above, the CO_2 level in the incubator should be set at 8% to maintain the appropriate pH. DMEM is not as well buffered as most medium formulations and consequently an alkaline pH is noted when only 5% CO_2 is used. This considerably delays cellular outgrowth and proliferation.
5. It is well known that different manufacturers have different processes by which plastic is treated to render it suitable for cell attachment. It has been noted that cellular outgrowth is more rapid on Falcon plates (Falcon, Los Angeles, CA) than on other brands. However, upon passage, there are no obvious differences between one brand and another.
6. The use of flasks for establishment of primary cultures is not recommended. Cellular outgrowth is dependent on the fragments remaining attached to the bottom of the dish. Upending flasks during feeding causes the fragments to become detached. Moving and feeding plates should be performed carefully to minimize disruption of the fragments from the bottom of the plate.

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